

# Activation of Nonselective Cation Channels by Physiological Cholecystokinin Concentrations in Mouse Pancreatic Acinar Cells

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**ABSTRACT** The activation of the nonselective cation channels in mouse pancreatic acinar cells has been assessed at low agonist concentrations using patch-clamp whole cell, cell-attached patch, and isolated inside-out patch recordings. Application of acetylcholine (ACh) (25–1,000 nM) and cholecystokinin (CCK) (2–10 pM) evoked oscillatory responses in both cation and chloride currents measured in whole cell experiments. In cell-attached patch experiments we demonstrate CCK and ACh evoked opening of single 25-pS cation channels in the basolateral membrane. Therefore, at least a component of the whole cell cation current is due to activation of cation channels in the basolateral acinar cell membrane. To further investigate the reported sensitivity of the cation channel to intracellular ATP and calcium we used excised inside-out patches. Micromolar  $\text{Ca}^{2+}$  concentrations were required for significant channel activation. Application of ATP and ADP to the intracellular surface of the patch blocked channel opening at concentrations between 0.2 and 4 mM. The nonmetabolizable ATP analogue, 5'-adenylylimidodiphosphate (AMP-PNP, 0.2–2 mM), also effectively blocked channel opening. The subsequent removal of ATP caused a transient increase in channel activity not seen with the removal of ADP or AMP-PNP. Patches isolated into solutions containing 2 mM ATP showed channel activation at micromolar  $\text{Ca}^{2+}$  concentrations. Our results show that ATP has two separate effects. The continuous presence of the nucleotide is required for operation of the cation channels and this action seems to depend on ATP hydrolysis. ATP can also close the channel and this effect can be demonstrated in excised inside-out patches when ATP is added to the bath after a period of exposure to an ATP-free solution. This action does not require ATP hydrolysis. Under physiological conditions hormonal stimulation can open the nonselective cation channels and this can be explained by the rise in the intracellular free  $\text{Ca}^{2+}$  concentration.

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## INTRODUCTION

$\text{Ca}^{2+}$ -activated nonselective cation channels were discovered in cultured cardiac cells (Colquhoun, Neher, Reuter, and Stevens, 1981) and subsequently found to be present in neuroblastoma (Yellen, 1982), freshly isolated mouse and rat pancreatic acinar cells (Maruyama and Petersen, 1982*a*), and lacrimal acinar cells (Marty, Tan, and Trautmann, 1984). Since then, these channels have been found in many other cell types (Partridge and Swandulla, 1988). In the pancreas these channels are closed in intact resting acinar cells but they can be activated by the secretagogues cholecystokinin (CCK) and acetylcholine (ACh) (Maruyama and Petersen, 1982*b*). In these experiments the agonists were applied to an area of the intact membrane not covered by the cell-attached patch-clamp pipette, demonstrating directly that CCK and ACh control the opening of the nonselective cation channels via an intracellular messenger, probably  $\text{Ca}^{2+}$  (Maruyama and Petersen, 1982*b*).

From the early patch-clamp studies on excised inside-out patches it appeared that  $\text{Ca}^{2+}$  in micromolar concentrations was needed on the inside of the plasma membrane to activate the nonselective cation channel (Colquhoun et al., 1981; Maruyama and Petersen, 1982*a, b*; Yellen, 1982), but Maruyama and Petersen (1984) later showed that the responsiveness of these channels to  $\text{Ca}^{2+}$  decreases with time after patch excision and just immediately after patch excision the  $\text{Ca}^{2+}$  sensitivity can be remarkably high.

A further complication arose when it became clear that adenosine triphosphate (ATP) acting on the inside of the membrane is able to close the  $\text{Ca}^{2+}$ -activated nonselective cation channels in an insulinoma cell line (Sturgess, Hales, and Ashford, 1986, 1987), in pancreatic acinar cells (Suzuki and Petersen, 1988), in kidney ascending Henle's loop cells (Paulais and Teulon, 1989), and in other epithelial cells (Cook, Poronnik, and Young, 1990; Gray and Argent, 1990). Considering that millimolar ATP concentrations are present in intact acinar cells (Matsumoto, Kanno, Seo, Murakami, and Watari, 1988), it is difficult to explain the mechanism by which channel activation occurs during receptor activation.

Although the experiments of Maruyama and Petersen (1982*b*) clearly showed that receptor activation can cause opening of the nonselective cation channels in intact pancreatic acinar cells, it is not clear whether this process occurs during stimulation with physiological levels of secretagogues. Maruyama and Petersen (1982*b*) used micropipette application of the peptide hormone cholecystokinin octapeptide (CCK8). In such experiments it is very difficult to estimate the actual hormone concentration at the receptor sites, but based on the effects of known concentrations of a CCK antagonist it was estimated that the CCK concentration could not be higher than 1 nM. This is, however, a pharmacological level since the physiological plasma concentration of CCK after a meal has been reported to rise to 5–20 pM (CCK8 equivalents) from a resting level of ~1 pM (CCK8 equivalents) (Walsh, 1987).

Our first aim in the experiments presented here was to determine whether physiological levels of CCK can evoke opening of the nonselective cation channels, and we now show that 5–10 pM CCK8 can induce such an effect. We therefore reinvestigated the effects of  $\text{Ca}^{2+}$  and ATP on channel gating and now report that although ATP does evoke acute channel closure when added to ATP-free solutions in

contact with the membrane inside, the continued presence of ATP is required in order for low  $\text{Ca}^{2+}$  concentrations to evoke channel opening.

Part of the work presented here has appeared in abstract form (Thorn, 1992).

## METHODS

### *Cell Preparation*

Isolated single mouse pancreatic acinar cells and small cell clusters were prepared by pure collagenase (Worthington Biochemical Corp., Freehold, NJ) digestion in the presence of trypsin inhibitor (2 mg/ml, Sigma type 11-S; Sigma Chemical Co., St. Louis, MO) as described previously (Osipchuk, Wakui, Yule, Gallacher, and Petersen, 1990).

### *Solutions*

The standard extracellular solution contained (mM): 140 NaCl, 4.7 KCl, 1.13  $\text{MgCl}_2$ , 11 glucose, 1  $\text{CaCl}_2$ , and 10 HEPES-NaOH, pH 7.2. In some whole cell current recording experiments the chloride concentration of this solution was lowered using Na acetate. The standard intracellular solution (pipette solution in whole cell experiments) contained (mM): 135 Kglutamate, 20 NaCl, 1.13  $\text{MgCl}_2$ , 0.1 EGTA, 2 ATP, and 10 HEPES-KOH, pH 7.2. For patch-clamp single channel current recording experiments on both cell-attached and isolated inside-out patches the pipette was filled with the standard extracellular solution. In isolated inside-out experiments the bathing solution (at the intracellular surface of the patch) contained (mM): 140 KCl, 5 NaCl, 1.13  $\text{MgCl}_2$ , 11 glucose, and 10 HEPES-KOH, pH 7.2.  $\text{Ca}^{2+}$ /EGTA buffers were used to set the free  $\text{Ca}^{2+}$  concentration of the solutions, and the  $\text{Ca}^{2+}$  chelating effects of the nucleotides were compensated for to keep the free  $\text{Ca}^{2+}$  concentration constant. The actual concentrations used were: 5  $\mu\text{M}$   $\text{Ca}^{2+}$  (2 mM EGTA, 1.942 mM  $\text{Ca}^{2+}$ ); 1  $\mu\text{M}$   $\text{Ca}^{2+}$  (2 mM EGTA, 1.721 mM  $\text{Ca}^{2+}$ ); 5  $\mu\text{M}$   $\text{Ca}^{2+}$ , 2 mM ATP (2 mM EGTA, 1.961 mM  $\text{Ca}^{2+}$ ); 5  $\mu\text{M}$   $\text{Ca}^{2+}$ , 4 mM ATP (2 mM EGTA, 1.99 mM  $\text{Ca}^{2+}$ ); and 5  $\mu\text{M}$   $\text{Ca}^{2+}$ , 4 mM ADP (2 mM EGTA, 1.95 mM  $\text{Ca}^{2+}$ ). The binding constants were all obtained from Fabiato and Fabiato (1979). It should be noted that free  $\text{Ca}^{2+}$  concentrations in the range used here are not very accurately determined by Ca/EGTA buffers since small errors in the pH measurements or in the amounts of EGTA and calcium added will cause relatively large deviations of the actual free  $\text{Ca}^{2+}$  concentrations from the calculated values. ATP, 5'-adenylylimido diphosphate (AMP-PNP), and ADP were all supplied by Sigma Chemical Co. Bath solution changes at the cell took less than 5 s. Changes were carried out through a row of polythene tubes (i.d. 0.5 mm; Portex Ltd., Hythe, Kent, UK) mounted on a manipulator (Prior Ltd.) and positioned in close proximity (1 mm) to the cell. An agar bridge bath electrode was used to minimize the formation of standing potentials when changing solution. In whole cell patch-clamp experiments a liquid junction potential of  $-7$  mV was produced due to unequal  $\text{Cl}^-$  ion concentrations in the bath and pipette. The quoted potentials were adjusted to account for this potential. The intracellular  $\text{Ca}^{2+}$  concentration in the ionomycin experiments was determined using Fura-2-loaded cells on a calibrated Joyce-Lobel Ltd. (Newcastle, UK) image analysis system (Toescu, Lawrie, Petersen, and Gallacher, 1992).

### *Experimental Protocol*

Experiments were all carried out at room temperature (20–25°C). Pipettes of 2–4 M $\Omega$  were pulled from microhematocrit (Assistant Micro-Haematocrit, No. 564) tubes and coated with Sylgard (Dow Corning Corp., Midland, MI) in single channel current recording experiments. Seals of  $>10$  G $\Omega$  were produced on the cell membrane and gentle suction or voltage pulses

often formed whole cell recordings as seen by an increase in capacitance and noise. Ionic equilibration between the cell and pipette contents was usually rapid (<20 s), judged by the stability of the current amplitudes recorded after establishment of the whole cell configuration. Any cells that showed slow changes in current amplitude or shape were rejected. Series resistance was measured by the compensation circuitry of the List EPC7 amplifier (List Medical Ltd., Darmstadt, Germany) and whole cell recordings were rejected if the series resistance was greater than 15 M $\Omega$ . The whole cell current records of Fig. 1, *A* and *B* were obtained by voltage steps from a holding potential of  $-48$  mV to a potential of 0 mV. Each potential was held for 150 ms and the stepping frequency was 3 Hz. At the resolution shown in Fig. 1 the two current traces obtained at the two holding potentials appear continuous. Single channel current recordings were all filtered at 1 kHz low-pass filter (Kemo Ltd., Beckenham, Kent, UK). The cell-attached patch records were held at a  $V_p$  of  $+70$  mV; the exact transpatch potential is unknown because of the existence of the cell resting potential.

#### *Single Channel Analysis*

Analysis of the single channel current cell-attached patch response to application of agonists was carried out by estimating the integral of the current response to obtain a measure of the mean current. In these experiments analysis of probability of channel opening was difficult because we did not know the number of channels in a patch. However, in some experiments on isolated inside-out patches we applied 1 mM  $\text{Ca}^{2+}$  in the absence of nucleotides, and observed a maximum number of simultaneously open channels. Any further increase in  $\text{Ca}^{2+}$  concentration failed to elicit an increase in the observed number of channels simultaneously open in the patch. We therefore measured the maximum number of channels open in a patch exposed to 1 mM  $\text{Ca}^{2+}$ ; in addition, we obtained a measure of the baseline current in the absence of channel opening. The channel open probability ( $P_o$ ) at the lower  $\text{Ca}^{2+}$  concentrations was then calculated from the single channel current obtained, as a proportion of the maximum possible current.

#### *Illustrations*

All current records were obtained by playing back the stored data from an FM tape recorder (Store 4DS; Racal Ltd., Hythe, UK) through the Cambridge Electronic Design (CED Ltd., Cambridge, UK) A/D converter. The data were then converted to a Hewlett Packard Graphics Language (HPGL)-compatible format using a program developed by Smith (1992).

## RESULTS

#### *Whole Cell Chloride and Cation Currents*

Whole cell recordings were made from isolated single pancreatic acinar cells. The chloride concentration was 150 mM in the bath and 22 mM in the pipette (substituting glutamate for chloride). Under these ionic conditions  $E_{\text{Cl}^-}$  is  $-48$  mV and  $E_{\text{cation}}$  is 0 mV. The membrane potential was held for 150 ms at  $-48$  mV and then stepped to 0 mV and held again for 150 ms. This protocol was repeated at a rate of 3 Hz and gave a measure of the nonselective cation and chloride currents, respectively.

In  $\sim 50$  cells, application of 2–10 pM CCK or 25–1,000 nM ACh elicited oscillatory and sustained cation and chloride currents. The relative amplitude of the two currents varied from cell to cell as previously shown by Randriamampita, Chanson, and Trautmann (1988) in experiments where 1  $\mu\text{M}$  ACh and 1 nM CCK were used.

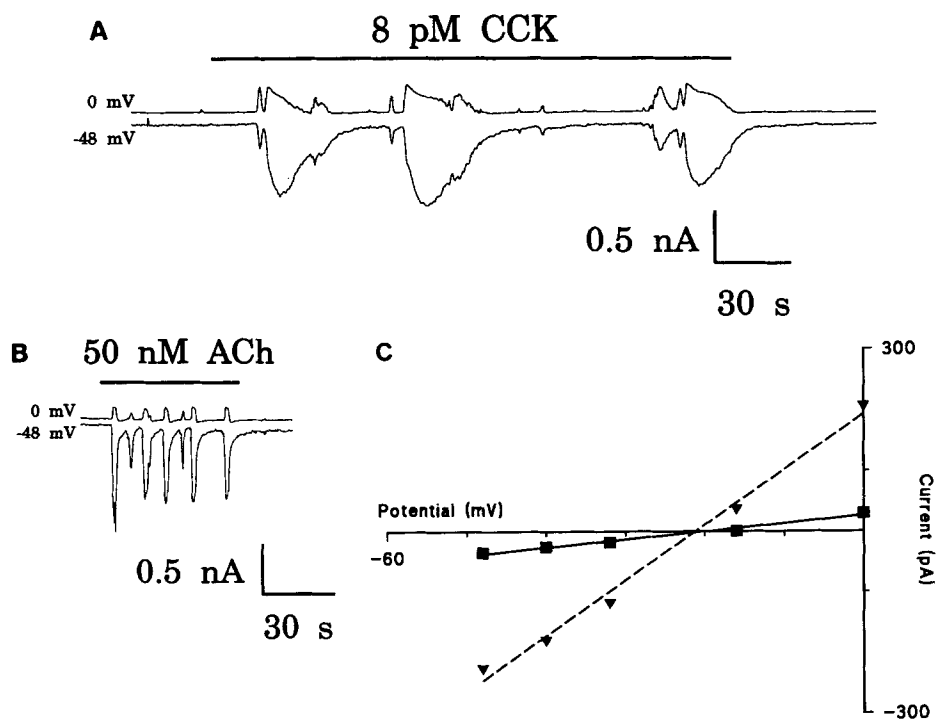


FIGURE 1. (A) Whole cell current records obtained in response to bath application of 8 pM CCK. A NaCl-rich bathing solution was used and the pipette was filled with a K-rich low chloride intracellular solution. The membrane potential was held at  $-48$  mV (lower trace) for 150 ms and then stepped to 0 mV (upper trace) for 150 ms, switching at a frequency of 3 Hz. On this time scale the individual steps are not resolved and the currents at the two voltages appear continuous. The current obtained at 0 mV (the reversal potential for the nonselective cation current) gave a measure of the chloride current. The current obtained at  $-48$  mV (the reversal potential of the chloride current) gave a measure of the nonselective cation current. It can be seen that CCK produces broad transients of both cation and chloride current. Each broad transient is preceded by a shorter spike. This type of pattern is commonly seen in response to these physiological doses of CCK and is sustained in the presence of CCK. (B) Whole cell current record obtained in response to bath application of 50 nM ACh. The voltage was stepped between  $-48$  and 0 mV, giving a measure of the cation and chloride currents, respectively. The relative amplitudes of the cation to chloride currents varied from cell to cell. In this particular case the cation current was larger than the chloride current. The current responses to low doses of ACh typically consisted of shorter oscillations than those for CCK, and as for CCK were maintained in the continued presence of agonist. (C) Current-voltage relationship obtained from a whole cell experiment before (squares) and after (triangles) application of 10 pM CCK. The points fitted by linear regression gave a slope conductance of 1.3 nS in control solutions and 9.2 nS in 10 pM CCK. The reversal potential of the response was  $-21$  mV.

Fig. 1A shows an oscillatory response to CCK in a physiological concentration (8 pM). Each large oscillation is preceded by a smaller transient response (Petersen, Toescu, and Petersen, 1991) seen in both the chloride (upper) and cation currents (lower). The cation current reached a peak in this experiment 9 s after the chloride

current. It was also characteristic in these whole cell recordings that the chloride current of the longer response returned to control levels some 10 s before the cation current. An additional observation was that the chloride current amplitude remained much the same for short and long transients, whereas the cation current increased in amplitude with the longer transients.

Low concentrations of ACh also elicited oscillations in both the cation and chloride currents as shown in Fig. 1 *B*. These oscillations were maintained in the continued presence of agonist, as with CCK, but are of shorter duration than those of CCK (Petersen et al., 1991). With these short oscillations the peak of the cation current was often observed after that of the chloride current, and the decay of the cation current was also longer. Fig. 1 *C* shows a current–voltage relationship obtained from a cell before (squares) and at the peak (triangles) of one of the repetitive transients induced by application of 10 pM CCK. The reversal potential at the peak of an agonist-induced response was  $-18.2 \pm 1.6$  mV (mean  $\pm$  SE). Voltage-clamp studies in mouse pancreatic segments with two intracellular microelectrodes have previously shown a reversal potential for the caerulein (CCK analogue) response of  $-20$  mV (Maruyama and Petersen, 1983).

Whole cell recording experiments were routinely performed with 2 mM ATP in the pipette, a concentration known to block nonselective channels (Sturgess et al., 1986; Suzuki and Petersen, 1988; Paulais and Teulon, 1989). It is, however, quite clear that in our experiments the nonselective current is not blocked (Fig. 1). In experiments carried out on different whole cells no significant difference in either the cation or chloride current amplitude or shape was observed with pipette ATP concentrations in the range 0–4 mM.

#### *CCK and ACh Activation of Single Nonselective Cation Channels in Intact Cells*

The whole cell current observations indicate activation of nonselective cation channels. We went on to record single channel currents from cell-attached patches to determine if this nonselective cation channel was the same as the one previously described in the basolateral membrane (Maruyama and Petersen, 1982*a, b*). In these experiments we used cells from small clusters. The advantage of clusters is that cell structure and polarity are well maintained, making it possible to identify the basolateral membrane. At low concentrations of agonist, ACh (25–100 nM) and CCK (5–10 pM) evoked opening of nonselective cation channels in cell-attached patches in ~50% of the experiments (8 of 14 for ACh and 9 of 20 for CCK). This is similar to the results in the whole cell recording configuration at these threshold agonist concentrations. Of the experiments showing effects of agonist stimulation, we rejected all those from the analysis in which there was no reversibility upon agonist removal, as this could indicate spontaneous excision of the membrane patch during the experiment. Some experiments could not be analyzed because of an unstable baseline. Fig. 2*A* shows single channel currents recorded at a  $V_p$  of +70 mV (transpatch potential unknown because it includes the cell resting potential), illustrating reversible channel activation after bath application of 5 pM CCK. The mean current activated by application of 5–10 pM CCK was  $3.05$  pA  $\pm$  0.9 (SE,  $n = 6$ ) and the mean current measured after the response was  $0.4$  pA  $\pm$  0.2 (SE,  $n = 6$ ). Fig. 2 *B* shows cation channel activation evoked by 100 nM ACh. The mean current activated

by 100 nM ACh was  $4.1 \text{ pA} \pm 1.3$  (SE,  $n = 5$ ) with a return after the response to  $0.2 \text{ pA} \pm 0.1$  (SE,  $n = 5$ ). In these cell-attached patch experiments the latency of the response was longer than in the whole cell configuration and channel openings persisted for some time after agonist removal. One possible explanation for these findings is that the omega shape formed by the membrane patch slows  $\text{Ca}^{2+}$

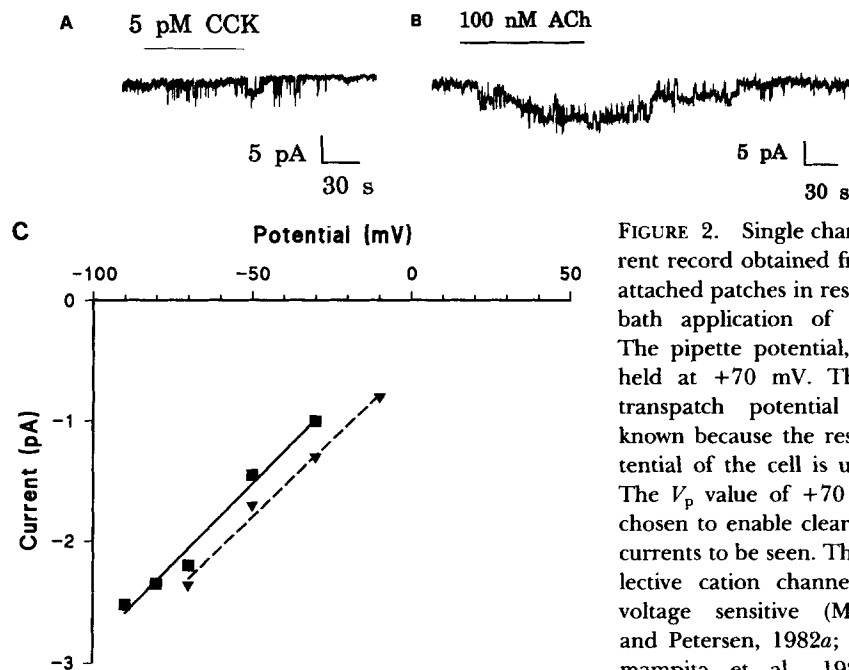


FIGURE 2. Single channel current record obtained from cell-attached patches in response to bath application of agonists. The pipette potential,  $V_p$ , was held at +70 mV. The exact transpatch potential is unknown because the resting potential of the cell is unknown. The  $V_p$  value of +70 mV was chosen to enable clear channel currents to be seen. The nonselective cation channel is not voltage sensitive (Maruyama and Petersen, 1982a; Randriamampita et al., 1988) and

therefore channel kinetics are not affected by holding potential. Current records were filtered at 1 kHz. The solutions of the pipette and the bath were NaCl rich with 1 mM  $\text{Ca}^{2+}$ . In the resting situation channel openings were rarely observed. (A) After application of 5 pM CCK to the bath, single inward channel currents are seen. In this experiment a maximum of two channels are seen to open simultaneously. The effect of CCK on channel openings was reversible. (B) Single channel current records obtained in response to bath application of 100 nM ACh. Open channel currents are downward. (C) Current-voltage relationships obtained from the single channel current amplitudes from cell-attached patches ( $n = 2-4$  for each point) in the presence of 100 nM ACh (triangles) and from isolated inside-out patches, 5  $\mu\text{M}$   $\text{Ca}^{2+}$ , K-rich solution in the bath (squares). The linear regression fit to the data (lines shown on the graph) represented a conductance of 27 pA and a reversal potential of 7 mV for isolated patches and a conductance of 26 pS and a reversal potential of 20 mV for the cell-attached patches.

equilibration in the submembrane compartment. The mean single channel conductance measured in cell-attached patches in the presence of 100 nM ACh (Fig. 2 C, triangles) and in excised inside-out patches (Fig. 2 C, squares) was  $26 \pm 2.2$  pS ( $n = 7$ , mean  $\pm$  SE). The reversal potential for the single channel currents in isolated patches with Na-rich solution in the pipette and K-rich solution in the bath was

approximately +7 mV (Fig. 2 C). Both the conductance and reversal potential are consistent with the nonselective cation channel properties previously described (Maruyama and Petersen, 1982a).

*Isolated Inside-out Patches: Ca<sup>2+</sup> Sensitivity*

Fig. 3 illustrates a typical experiment ( $n = 13$  cells) on the Ca<sup>2+</sup> sensitivity of the nonselective cation channel. All patches for this series of experiments were obtained from the acinar cell basolateral membrane using small cell clusters as explained previously. The patches were held at a membrane potential of -70 mV and excised in a KCl-rich bath solution containing 1.13 Mg<sup>2+</sup> and ~1 mM Ca<sup>2+</sup>. The channel open probability ( $P_o$ ) in 1 mM Ca<sup>2+</sup> solution was calculated at  $0.648 \pm 0.058$  ( $n = 5$ ,

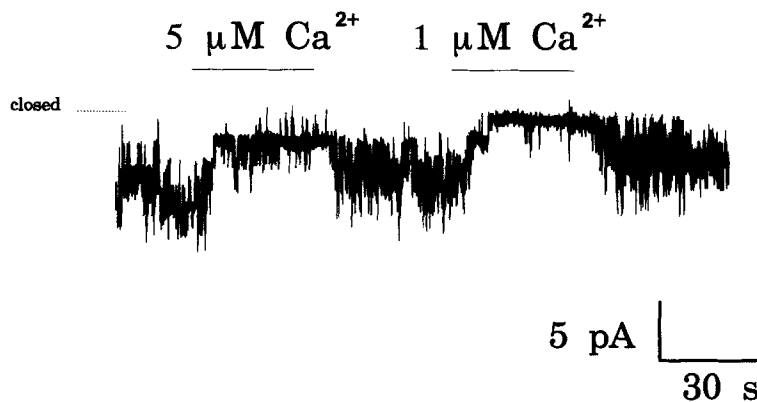


FIGURE 3. Single channel current recording obtained from an inside-out patch isolated into a KCl-rich bathing solution containing 1 mM Ca<sup>2+</sup>. The pipette solution was NaCl rich. The patch was held at -70 mV and channel openings were downward. Channel activity decreases when the Ca<sup>2+</sup> concentration is reduced to ~5 μM Ca<sup>2+</sup> at the intracellular surface of the patch. The maximum number of simultaneously open channels is decreased from five in 1 mM Ca<sup>2+</sup> to three. Reduction of the bath Ca<sup>2+</sup> concentration to 1 μM decreases channel activity considerably, with only one channel observed to open at a low frequency. The effects of lowered Ca<sup>2+</sup> concentrations are rapid and reversible.

mean  $\pm$  SE). Lower concentrations of Ca<sup>2+</sup> were applied to the intracellular surface of the patch via a rapid local perfusion system.  $P_o$  decreased at a Ca<sup>2+</sup> concentration of ~5 μM to  $0.177 \pm 0.038$  ( $n = 7$ , mean  $\pm$  SE) and at a concentration of ~1 μM Ca<sup>2+</sup>  $P_o$  was  $0.024 \pm 0.005$  ( $n = 6$ , mean  $\pm$  SE).

*Isolated Inside-out Patches: Effect of Intracellular Nucleotides*

Previous work on isolated inside-out patches has demonstrated block of cation channel opening by ATP applied to the intracellular surface of the patch (Suzuki and Petersen, 1988) and low Ca<sup>2+</sup> sensitivity (Maruyama and Petersen, 1984). We investigated these phenomena in more detail in light of the demonstrated activation of the nonselective cation current in experiments on intact cells and in whole cell experiments with low agonist concentrations.



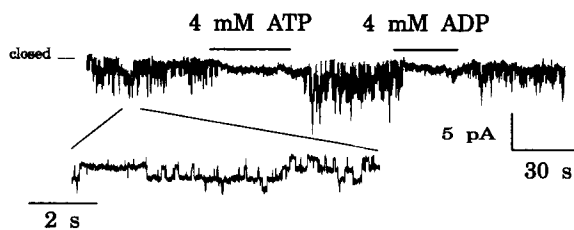


FIGURE 4. Single channel current records from an inside-out patch excised in KCl-rich solution with  $5 \mu\text{M Ca}^{2+}$ , held at a membrane potential of  $-70 \text{ mV}$ . The pipette was filled with NaCl-rich solution containing  $1 \text{ mM Ca}^{2+}$ . The nucleotides ADP and ATP ( $4 \text{ mM}$ ) were applied to the intracellular surface of the patch. Both caused a rapid, reversible inhibition of channel opening. In these and all subsequent experiments the  $\text{Ca}^{2+}$  chelating effects of the nucleotides used were compensated for by adjustment of the  $\text{Ca}^{2+}$ /EGTA ratio so that the free  $\text{Ca}^{2+}$  remained approximately constant.

In these experiments the inside of the patch membrane was in contact with a KCl-rich solution containing  $\sim 5 \mu\text{M Ca}^{2+}$  and nucleotides were applied to the intracellular surface of the membrane. In all cases we attempted to keep the  $\text{Ca}^{2+}$  concentration approximately constant by adjusting the EGTA/ $\text{Ca}^{2+}$  ratio to take into account the  $\text{Ca}^{2+}$  chelating action of the nucleotides. Fig. 4 illustrates a typical experiment ( $n = 24$ ) on one patch where ADP and ATP were successively applied to the intracellular surface of an isolated inside-out patch. Both nucleotides promoted a rapid, reversible, and complete block of channel opening. To investigate the possible involvement of phosphorylation events we went on to study the effect of the nonhydrolyzable ATP analogue AMP-PNP. This agent has previously been shown to block nonspecific cation channels studied in other preparations (Sturgess et al., 1986; Paulais and Teulon, 1989).

Fig. 5 shows a typical result of the application of AMP-PNP ( $2 \text{ mM}$ ;  $n = 5$  cells) and ATP ( $2 \text{ mM}$ ;  $n = 10$ ). Both caused a rapid and reversible channel closure. This result indicates that the nucleotide-mediated block of channel opening is not the product of protein phosphorylation. One consistent and novel finding was a transient increase in

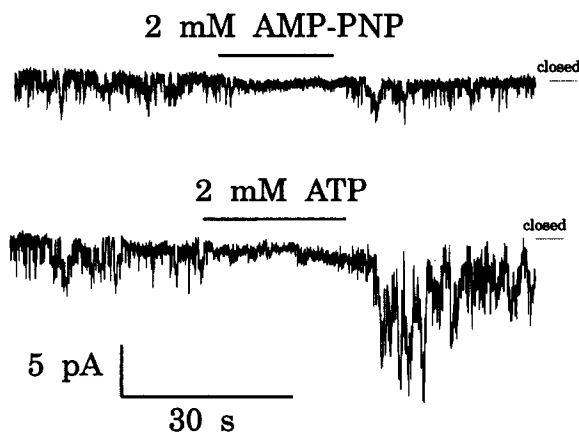


FIGURE 5. Single channel current records obtained under the same conditions as in Fig. 4. The bathing solution was KCl-rich,  $5 \mu\text{M Ca}^{2+}$ , the pipette solution was NaCl-rich,  $1 \text{ mM Ca}^{2+}$ . Bath application of  $2 \text{ mM AMP-PNP}$  (upper panel) and  $2 \text{ mM ATP}$  (lower panel) both promoted a rapid, reversible channel block. After removal of ATP a rebound effect was seen with a transient increase in the number of simultaneously open channels observed. This rebound was never observed after application of ADP or AMP-PNP.

the number of open channels after the removal of ATP (Figs. 4 and 5). This effect was never seen after the application of ADP or AMP-PNP (Figs. 4 and 5). ATP can therefore evoke acute channel closure, but is also able to maintain channels in an operational state. The first effect can be produced by ADP and AMP-PNP, whereas the latter cannot.

*Evidence for a Tonic Influence of ATP*

We tested the hypothesis that ATP may be exerting a long-term influence on channel opening. In these experiments we applied ionomycin ( $0.2 \mu\text{M}$ ) to a cell while recording from a cell-attached patch. The ionomycin was present in a solution containing  $\sim 5 \mu\text{M Ca}^{2+}$  and gave in a separate series of experiments a measured rise

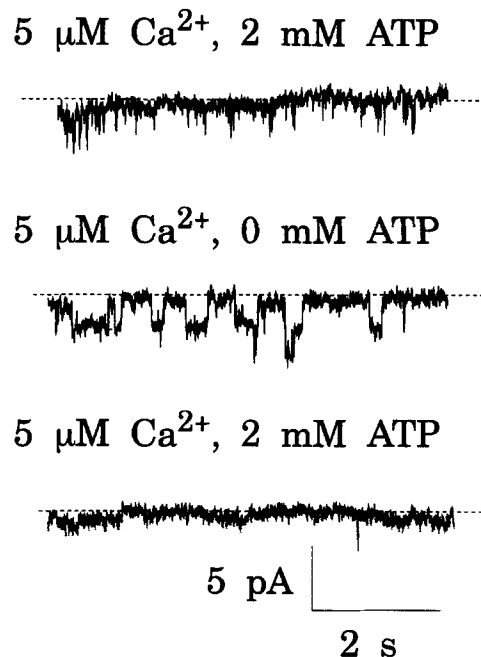


FIGURE 6. Single channel current recordings obtained from an inside-out patch isolated in a KCl-rich bathing solution containing  $\sim 5 \mu\text{M Ca}^{2+}$  and 2 mM ATP (*top trace*). The pipette solution was NaCl-rich, 1 mM  $\text{Ca}^{2+}$ . The membrane potential was  $-70 \text{ mV}$  and channel openings were downward. The middle trace shows the effect of removal of ATP in the continued presence of the same  $\text{Ca}^{2+}$  concentration ( $\sim 5 \mu\text{M}$ ). Channel activity is seen to increase. The bottom trace was obtained later in the same experiment and shows the effect of the subsequent return to a bathing solution containing 2 mM ATP and  $\sim 5 \mu\text{M Ca}^{2+}$ . In this case, in the same patch and with the same solution as used in the upper trace no channel openings were observed.

in the free intracellular  $\text{Ca}^{2+}$  concentration from 75 to 1,469 nM ( $n = 7$ ). All patches showed opening of the nonselective channel after addition of ionomycin to the cell ( $n = 4$ ), with an increase in mean current from  $0.07 \pm 0.04 \text{ pA}$  (mean  $\pm$  SE) in control solution to  $4.73 \pm 1.3 \text{ pA}$  (mean  $\pm$  SE) in a solution containing ionomycin and  $5 \mu\text{M Ca}^{2+}$ . In another series of experiments ( $n = 6$ ) patches were excised in solutions containing 2 mM ATP. In this way the intracellular surface of the patch was continuously exposed to ATP-containing solutions. In all patches nonselective cation channel openings were observed during initial exposure of the intracellular surface of the patch to  $\sim 5 \mu\text{M Ca}^{2+}$ , even in the presence of 2 mM ATP (Fig. 6). The calculated probability of channel opening in the solution containing  $\sim 5 \mu\text{M Ca}^{2+}$  and 2 mM ATP was  $0.341 \pm 0.126$  ( $n = 3$ , mean  $\pm$  SE) and in  $\sim 1 \mu\text{M Ca}^{2+}$  and 2 mM ATP it

was  $0.038 \pm 0.014$  ( $n = 5$ , mean  $\pm$  SE). Also shown in Fig. 6 is the effect of the subsequent removal of ATP. Fig. 6 (lower panel) illustrates the currents obtained later in the experiment after returning to ATP-containing solution. No channel openings are now observed.

#### DISCUSSION

The experiments presented in this paper show that a physiological CCK concentration (Walsh, 1987) evokes opening not only of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels (Fig. 1) (Petersen et al., 1991) but also of  $\text{Ca}^{2+}$ -dependent nonselective cation channels. In the case of the cell-attached patch experiments the cell remains intact and the demonstration of nonselective cation channel opening in response to physiological concentrations of CCK (5–10 pM) (Fig. 2) is the most persuasive evidence to date of a role for this channel in normal secretory events in these cells. The experiments using isolated inside-out patches (Fig. 4) highlight the long-standing dilemma that under what are apparently physiological concentrations of nucleotides the nonselective channel is closed even at high intracellular  $\text{Ca}^{2+}$  concentrations (Suzuki and Petersen, 1988). However, the demonstration that the channel shows an increase in activity after exposure to ATP but not ADP or a nonhydrolyzable analogue (Fig. 5) indicates that there must be another site available for channel modulation apart from the calcium binding site and the binding site involved in nucleotide-evoked block. The data obtained from excised patches in bath solutions containing ATP (Fig. 6) show that the channel can open in the presence of micromolar  $\text{Ca}^{2+}$  concentrations. It therefore seems possible that ATP binding regulates the channel in the intact cell and allows activation by physiological  $\text{Ca}^{2+}$  signals.

#### *Whole Cell Currents*

The response to CCK (Fig. 1A) clearly shows an asymmetry between the chloride and cation currents. The chloride current reaches a peak before the cation current and then decays at a faster rate than the cation current. This has been observed before in the single transient responses to high agonist concentrations recorded by both Kasai and Augustine (1990) and Randriamampita et al. (1988). We show here, for the first time, that the same current patterns are seen in oscillatory responses to the application of physiological agonist concentrations. The different rates of inactivation of the two currents have been interpreted by Kasai and Augustine (1990) either as chloride channel inactivation in the continued presence of  $\text{Ca}^{2+}$  or as a decay of the  $\text{Ca}^{2+}$  concentration in the luminal pole behind the basolaterally directed  $\text{Ca}^{2+}$  wave. Randriamampita et al. (1988) have interpreted the data on the basis of the different  $\text{Ca}^{2+}$  sensitivities of the chloride and cation currents. In this way the agonist-induced rise in cytoplasmic  $\text{Ca}^{2+}$  concentration causes activation of the chloride channel first because of the lower threshold of activation of these channels. Previous data suggest a high (greater than micromolar) threshold for  $\text{Ca}^{2+}$  of the cation channel in excised patches (Maruyama and Petersen, 1982a), but it has also been found that in saponin-permeabilized cells a  $\text{Ca}^{2+}$  concentration as low as  $5 \times 10^{-8}$  M is sufficient to promote channel opening (Maruyama and Petersen, 1984). On the basis of our work we can conclude that low agonist concentrations are able to induce oscillations in both the nonselective cation current and the chloride current.

*Cell-attached Patch Recordings*

These experiments confirm the results obtained in the whole cell configuration indicating activation of the cation current at low agonist concentrations. Since the patch pipette was attached to the basolateral membrane, this indicates that even the brief oscillatory responses involve activation of nonselective cation channels in this part of the cell. We cannot, however, rule out the possibility that nonselective cation channels are also located in (Cook et al., 1990; Gray and Argent, 1990) or very close to the luminal pole. Activation of the basolaterally located nonselective cation channels using supramaximal agonist concentrations has already been demonstrated (Maruyama and Petersen, 1982*b*). It is now clear from the experiments presented here that the nonselective cation channels are activated at CCK concentrations found in the circulation after a meal (Walsh, 1987) and therefore may be involved in the control of the normal secretory process. We interpret the slow decay of channel opening in cell-attached patches after agonist activation to be a result of restricted access of  $\text{Ca}^{2+}$  to the submembrane area due to the omega shape of the patch (Sakmann and Neher, 1983). This would lead to a slow decay of  $\text{Ca}^{2+}$  in the region immediately below the channels and extend the period of channel activation.

*Isolated Inside-out Patches*

These experiments illustrate the  $\text{Ca}^{2+}$  sensitivity of the isolated patches in the absence of ATP and show that micromolar  $\text{Ca}^{2+}$  concentrations are required for channel activation. An investigation of the nucleotide sensitivity of the channel revealed block of channel opening down to concentrations of 0.1 mM ATP. In addition, both ADP and the nonhydrolyzable AMP-PNP effectively block. This indicates a relatively unselective nucleotide binding site for the inhibition of channel opening. The transient channel activation that follows ATP removal most probably involves binding at a different site, and the lack of effect of AMP-PNP or ADP supports the suggestion that it involves a phosphorylation step. Nucleotides have also been shown to block the nonselective cation channel of an insulinoma cell line (Sturgess et al., 1986) and, as with the pancreatic acinar cells, AMP-PNP was effective as a blocking agent. Extensive studies have been carried out on the nucleotide sensitivity of the ATP-sensitive,  $\text{K}^{+}$ -selective channel (Petersen and Findlay, 1987; Ashcroft, 1988). In insulin-secreting cells the  $\text{K}_{\text{ATP}}$  channel is blocked by ATP, but ADP has a variable effect (Dunne and Petersen, 1986; Kakei, Kelly, Ashcroft, and Ashcroft, 1986). When applied alone ADP is inhibitory; however, when applied together with ATP it relieves the channel block. This effect was not seen in experiments on the nonselective cation channel of pancreatic acinar cells since ADP applied on top of ATP had no effect (data not shown). The ATP stimulation of channel opening seen in pancreatic acinar cells is also observed in the  $\text{K}_{\text{ATP}}$  channels of the insulin-secreting cells. ATP is able to restore the activity in patches where the channels have been allowed to run down after excision from the cell and this has been interpreted as being due to protein phosphorylation (Petersen and Findlay, 1987).

The experiments using patches isolated in the continuous presence of ATP at the intracellular surface of the patch still show channel opening, an effect that is

subsequently lost when ATP is readmitted after a period in which no ATP was present. One explanation could be a rundown or loss of a regulatory component either present in the cytoplasm or membrane bound. Intracellular ATP concentrations of rat pancreatic acinar cells have been shown to be maintained at millimolar concentrations in the presence of agonists (Matsumoto et al., 1988). Therefore, under physiological conditions the nonselective cation channels would always be exposed to high levels of ATP. The nonselective cation channel is activated by ionomycin in the cell-attached patches where the intracellular  $\text{Ca}^{2+}$  concentration is  $1.5 \mu\text{M}$  and the normal ATP concentration would be expected to be maintained. This  $\text{Ca}^{2+}$  concentration represents a value close to physiological significance during stimulation of the cell.

#### *Physiological Role of the Cation Channel*

In the mouse pancreatic acinar cells the physiological function of the nonselective cation channel remains unclear. One proposed function is a role as a calcium influx pathway (Petersen and Maruyama, 1983a, b; Sasaki and Gallacher, 1990). Another possibility is a role in fluid secretion. In contrast to other acinar cell types, the mouse and rat pancreatic acinar cells do not possess the  $\text{Ca}^{2+}$ - and voltage-dependent high conductance K channel important in providing the outward driving force for the secretion of chloride into the lumen (Petersen and Gallacher, 1988). The standard model for acinar fluid secretion (Petersen and Gallacher, 1988) therefore cannot apply to mouse and rat pancreatic acinar cells (Petersen, 1992). Our data show that physiological concentrations of secretagogues can evoke oscillatory opening of the nonselective cation channels, a critically important step in the push-pull model of fluid secretion proposed for these cells by Kasai and Augustine (1990). In cultured epithelial cells (Cook et al., 1990) and pancreatic duct cells (Gray and Argent, 1990) nonselective cation channels have been found in both the basolateral membrane and the apical cell membrane. We do not know whether these channels are present in the luminal membranes of the acinar cells, but if they are they could play a role in the  $\text{Na}^+$  recirculation through the cells and the paracellular pathway which has been proposed to explain isotonic fluid secretion (Ussing and Eskesen, 1989).

We have demonstrated for the first time that the  $\text{Ca}^{2+}$ -sensitive nonselective cation channel is opened in intact cells by physiological agonist concentrations. We have explored the  $\text{Ca}^{2+}$  and nucleotide sensitivity of the channel in isolated inside-out patches and confirmed that ATP blocks channel openings. Our experiments indicate that the ATP block is not seen when patches are isolated in the continuous presence of ATP. The  $\text{Ca}^{2+}$  sensitivity under these circumstances may be close to the physiological range.

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## REFERENCES

- Ashcroft, F. M. 1989. Properties and functions of ATP-sensitive K-channels. *Annual Review of Neuroscience*. 11:97–118.
- Colquhoun, D., E. Neher, H. Reuter, and C. F. Stevens. 1981. Inward current channels activated by intracellular  $\text{Ca}^{2+}$  in cultured cardiac cells. *Nature*. 294:752–754.
- Cook, D. I., D. Poronnik, and J. A. Young. 1990. Characterization of a 25-pS nonselective cation channel in a cultured secretory epithelial cell line. *Journal of Membrane Biology*. 114:37–52.
- Dunne, M. J., and O. H. Petersen. 1986. Intracellular ADP activates  $\text{K}^+$  channels that are inhibited by ATP in an insulin-secreting cell line. *FEBS Letters*. 208:59–62.
- Fabiato, A., and F. Fabiato. 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *Journal of Physiology (Paris)*. 75:463–505.
- Gray, M. A., and B. E. Argent. 1990. Non-selective cation channel on pancreatic duct cells. *Biochimica et Biophysica Acta*. 1029:33–42.
- Kakei, M., R. J. Kelly, S. J. H. Ashcroft, and F. M. Ashcroft. 1986. The ATP-sensitivity of  $\text{K}^+$  channels in rat pancreatic B-cells is modulated by ADP. *FEBS Letters*. 208:63–66.
- Kasai, H., and G. J. Augustine. 1990. Cytosolic  $\text{Ca}^{2+}$  gradients triggering unidirectional fluid secretion from exocrine pancreas. *Nature*. 348:735–738.
- Marty, A., Y. P. Tan, and A. Trautmann. 1984. Three types of calcium-dependent channel in rat lacrimal glands. *Journal of Physiology*. 357:293–325.
- Maruyama, Y., and O. H. Petersen. 1982a. Single channel currents in isolated patches of plasma membrane from basal surface of pancreatic acini. *Nature*. 299:159–161.
- Maruyama, Y., and O. H. Petersen. 1982b. Cholecystokinin activation of single channel currents is mediated by internal messenger in pancreatic acinar cells. *Nature*. 300:61–63.
- Maruyama, Y., and O. H. Petersen. 1983. Voltage clamp study of stimulant-evoked currents in mouse pancreatic acinar cells. *Pflügers Archiv*. 399:54–62.
- Maruyama, Y., and O. H. Petersen. 1984. Single calcium-dependent cation channels in mouse pancreatic acinar cells. *Journal of Membrane Biology*. 81:83–87.
- Matsumoto, T., T. Kanno, Y. Seo, M. Murakami, and H. Watari. 1988. Phosphorus nuclear magnetic resonance in isolated perfused rat pancreas. *American Journal of Physiology*. 254:G575–G579.
- Osipchuk, Y., M. Wakui, D. I. Yule, D. V. Gallacher, and O. H. Petersen. 1990. Cytoplasmic  $\text{Ca}^{2+}$  oscillations evoked by receptor stimulation, G protein activation, internal application of inositol trisphosphate or  $\text{Ca}^{2+}$ : simultaneous microfluorimetry and  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  current in single pancreatic acinar cells. *EMBO Journal*. 9:697–704.
- Partridge, L. D., and D. Swandulla. 1988. Calcium-activated non-specific cation channels. *Trends in Neurosciences*. 11:69–72.
- Paulais, M., and J. Teulon. 1989. A cation channel in the thick ascending limb of Henle's loop of the mouse kidney: inhibition by adenine nucleotides. *Journal of Physiology*. 413:315–327.
- Petersen, C. C. H., E. C. Toescu, and O. H. Petersen. 1991. Different patterns of receptor-activated cytoplasmic  $\text{Ca}^{2+}$  oscillations in single pancreatic acinar cells: dependence on receptor type, agonist concentration and intracellular  $\text{Ca}^{2+}$  buffering. *EMBO Journal*. 10:527–533.
- Petersen, O. H. 1992. Stimulus-secretion coupling: cytoplasmic calcium signals and the control of ion channels in exocrine acinar cells. *Journal of Physiology*. 448:1–51.
- Petersen, O. H., and I. Findlay. 1987. Electrophysiology of the pancreas. *Physiological Reviews*. 67:1054–1116.
- Petersen, O. H., and D. V. Gallacher. 1988. Electrophysiology of pancreatic and salivary acinar cells. *Annual Reviews of Physiology*. 50:65–80.

- Petersen, O. H., and Y. Maruyama. 1983a. What is the mechanism of the calcium influx to pancreatic acinar cells evoked by secretagogues. *Pflügers Archiv*. 396:82–84.
- Petersen, O. H., and Y. Maruyama. 1983b. Cholecystokinin and acetylcholine activation of single-channel currents via second messenger in pancreatic acinar cells. In *Single Channel Recording*. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York. 425–435.
- Randriamampita, C., M. Chanson, and A. Trautmann. 1988. Calcium and secretagogues-induced conductances in the rat exocrine pancreas. *Pflügers Archiv*. 411:53–57.
- Sakmann, B., and E. Neher. 1983. Geometric parameters of pipettes and membrane patches. In *Single Channel Recording*. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York. 37–51.
- Sasaki, T., and D. V. Gallacher. 1990. Extracellular ATP activates receptor operated cation channels in mouse lacrimal acinar cells to promote calcium influx in the absence of phosphoinositide metabolism. *FEBS Letters*. 264:130–134.
- Smith, P. M. 1992. Patch-clamp whole cell pulse protocol measurements using a microcomputer. *Journal of Physiology*. 446:72P.
- Sturgess, N. C., C. N. Hales and M. L. J. Ashford. 1986. Inhibition of a calcium-activated, non-selective cation channel, in a rat insulinoma cell line, by adenine derivatives. *FEBS Letters*. 208:397–400.
- Sturgess, N. C., C. N. Hales, and M. L. J. Ashford. 1987. Calcium and ATP regulate the activity of a non-selective cation channel in a rat insulinoma cell line. *Pflügers Archiv*. 409:607–615.
- Suzuki, K., and O. H. Petersen. 1988. Patch-clamp study of single-channel and whole-cell  $K^+$  current in guinea pig pancreatic acinar cells. *American Journal of Physiology*. 255:G275–285.
- Thorn, P. 1992. Activation of non-selective cation channels by physiological cholecystokinin concentrations in mouse pancreatic acinar cells. *Journal of Physiology*. 446:129P.
- Toescu, E. C., A. M. Lawrie, O. H. Petersen, and D. V. Gallacher. 1992. Spatial and temporal distribution of agonist-evoked cytoplasmic  $Ca^{2+}$  signals in exocrine acinar cells analysed by digital image microscopy. *EMBO Journal*. 11:1623–1629.
- Ussing, H. H., and K. Eskesen. 1989. Mechanism of isotonic water transport in glands. *Acta Physiologica Scandinavica*. 136:443–454.
- Walsh, J. H. 1987. Cholecystokinin. In *Physiology of the Gastrointestinal Tract*. 2nd ed. L. R. Johnson, editor. Raven Press, New York. 195–206.
- Yellen, G. 1982. Single  $Ca^{2+}$ -activated non-selective cation channels in neuroblastoma. *Nature*. 296:357–359.